STUDIES ON VITAL STAINING

III. The Simultaneous Ingestion of Two Dyestuffs by Phagocytes. The Question of "Blockade of the Reticulo-Endothelial System"

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INTRODUCTION

In previous articles of this series (1) it has been shown that brilliant vital red is relatively non toxic to dogs when given intravenously in rather large amounts. The dye is removed from the plasma, and prior to its gradual elimination from the body is stored in various tissues, mainly in the large groups of phagocytic cells sometimes spoken of collectively as the "reticulo-endothelial system." It was shown that the dye passes from plasma to tissues for many hours, but eventually an equilibrium point is approached, and from this time on dye leaves the circulation very slowly. This virtual cessation of phagocytic activity may appear long before all of the dye has left the circulating plasma, and this is true especially when large amounts of dye are injected. It is in these very cases that the tissue phagocytes themselves are most deeply stained. We concluded that the cessation in phagocytic activity is the result of the partial filling of these cells with dye, but it was possible to show that on injecting more dye the phagocytes will at once resume their activity, and for a time dye leaves the circulation rather rapidly. Quite evidently this injection simply disturbed the balance between dye in plasma and dye in tissues, and the balance could not be restored until part of the newly injected dye had passed from plasma to tissues. This state of balance illustrates a curious form of inactivity on the part of living cells. This inactivity is clearly associated with the presence of dye in the tissues. This is not an inhibition or "blockade" in the ordinary sense of the word, for if we

disturb the equilibrium by injecting more dye we find that the dye-laden cells show a surprising ability to take up the newly offered dye. The evidence indicates that the phagocytic response toward new dye may equal that of normal unstained tissues.

It is of great interest to investigate the reactions of these tissues toward the introduction of a second dyestuff—for analytical convenience a blue dye. We may hope to ascertain whether the presence of the red dye in the tissues will inhibit the entrance of the blue one and whether the final partition of the blue one between plasma and tissues will be influenced by the presence of the red dye in the body. Such studies involve the existence of what we might term "cross inhibition," and it is this form of inhibition to which most workers refer when they speak of "blockade." We may apply the term "specific inhibition" to the concept that tissues laden with one particular dye are so affected that they take up this particular dye not at all or with distinct reluctance. In our own studies such "specific" inhibition was clearly demonstrable only in the sense of an equilibrium between dye in tissues and in plasma. It was impossible to demonstrate "specific" inhibition in the larger sense which involves reluctance of tissues to take up subsequent offerings of this particular dye.

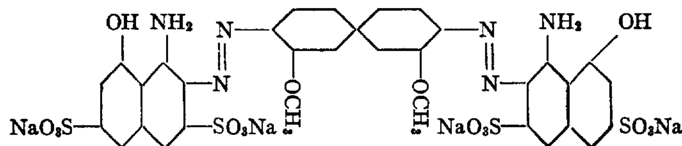
The experiments about to be reported deal with the question of "cross" inhibition, and for purposes of investigation we have injected aqueous solutions of brilliant vital red and of Niagara sky blue. These two dyes belong to the same general class. With the exception of color both have many physical properties in common. Both have certain colloidal properties. In the matter of diffusibility they are rather closely similar. The general similarity in chemical constitution is shown by the structural formulae given below. From these numerous points of similarity one might anticipate finding many points of similarity in physiological behavior also, and we need not be surprised that the two dyes may be demonstrated in the same cells, either side by side in granular form, or, under certain circumstances fused intimately to form purple granules within the phagocytes (2). Here there can be no question that the same group of cells is responsible for removing much of the dye from the plasma—a questionable assumption when widely differing substances are used in the study of "block-

ade." Highly colloidal dyestuffs such as brilliant vital red and Niagara sky blue have much to recommend them in studies of the sort here reported. The relatively slow elimination from the blood stream makes it easy to follow the process by quantitative methods. No doubt the desirability of such studies with dyes so similar in their general properties has occurred to many, but without the spectrophotometric method of analysis (3), it is well nigh impossible to make analysis of the purple plasma which results from the admixture of the two dyes.

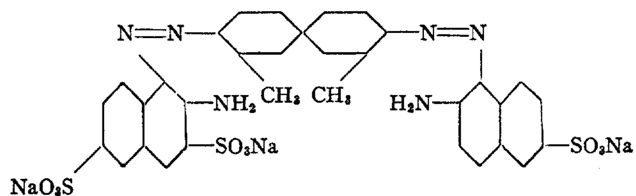
The experiments to follow give no evidence to show that the presence of one dye within the tissues influences in any manner the ability of the cells to take up and store another, and this is true regardless of whether the two dyes are given simultaneously or in sequence. Some of the dogs were vitally stained with repeated daily injections of brilliant vital red, after which a measured quantity of Niagara sky blue was injected, and the concentration of this dye in the blood stream determined over a period of several days, and it was found that it left the blood stream at almost identically the normal control rate. In other experiments the two dyes were injected simultaneously, and here again it will be shown that within limits of experimental error the tissues take up the blue dye at the normal rate, and this despite the fact that they were engaged at the same time in taking up the red dye also. Furthermore in the course of 72–96 hours the blue dye is leaving the blood stream very slowly—evidence of a state of equilibrium. The amount of blue dye now in circulation is almost identical with that observed in control experiments where no red dye was given. This shows that the final partition ratio between plasma and tissue is not influenced by the fact that red dye is present also. Presumably in these experiments the red dye is also distributed between plasma and tissues in a manner normally characteristic for that dye. Unfortunately the absorption curves of the two dyes are such that quantitative spectroscopic measurement of the red dye in the purple plasma is not so accurate as in the case of the blue one, and we found it impractical to follow the elimination of the red dye, though attempts along this line have shown that there is certainly no gross deviation from normal in the elimination of the latter.

Methods

Normal adult dogs maintained on a mixed diet were used. As in the previous experiments, feeding hours were so arranged that the plasma would show no lipemia during the hours when samples were taken for dye analysis. The two dyes used, brilliant vital red and Niagara sky blue, were obtained from the National Aniline and Chemical Company. The trade names for these dyes are those given them by that company. It is said that brilliant vital red corresponds chemically to brilliant congo R, rouge congo brillante R, brillantdianilrot R and azidinscharlach R of other manufacturers. It is Schultz no. 370 and chemically is 1 mol of tolidine combined with 1 mol of β napthylamine, 3, 6, disulphonic acid and 1 mol of β naphthylamine, 6 monosulphonic acid. Niagara sky blue corresponds to Schultz no. 426 and chemically is 1 mol of dianisidine combined with 2 mols of H acid. It thus corresponds to dyes marketed by various firms under the names of diaminreinblau, bleu pur diamine, dianiblau H 6 G, oxaminreinblau 5 B, direktblau R B A, etc. The structural formulae are thought to be:



Niagara Sky Blue



Brilliant Vital Red

For purposes of injection these dyes were made up in a two per cent aqueous solution.

The blood samples were collected in well-vaselined "record" syringes and every precaution was taken to avoid hemolysis. The samples of blood were run into 15 cc. graduated hematocrit tubes containing 2 cc. of a 1.6 per cent solution of sodium oxalate to prevent clotting. After centrifugalization the amount of oxalated plasma in the tube was read off, thus enabling one to correct for the amount of oxalate solution present. The amount of dye in the plasma was determined by means of the spectrophotometer by a method (3) which allows quantitative analysis of each dye even when the two dyes are present together in the plasma.

EXPERIMENTAL

The data here reported were obtained from similar experiments carried out on 4 different dogs. The individual protocols are presented separately (experiments 45–48 incl.). Table 31 shows the results obtained in the individual cases. One of the experiments (experiment 46) was incomplete, and for this reason it was not used in making up the averages which are shown graphically in Charts 3A and 3B. For ease in presentation we shall refer chiefly to these curves where the progress of dye elimination can be seen at a glance. A careful study of the individual experiments shows minor variations, but all are alike in all essential respects.

Experiment 45. Dog 25–29. Male, Collie, 22 kg.

January 18, 1927. Twenty-four cubic centimeters of a 2 per cent solution of Niagara sky blue injected intravenously. At various intervals samples of blood were removed for analysis.

February 13, 1927. An intravenous injection consisting of 24 cc. of 2 per cent Niagara sky blue and 24 cc. of brilliant vital red was followed over a period of 4 days by frequent analysis of the plasma for Niagara sky blue. A sample of plasma taken immediately before making the double dye injection showed that the plasma had become free of the dye injected 3 weeks previously.

May 1 to 9th inclusive, daily injections of 24 cc. of a 2 per cent solution of brilliant vital red.

May 11. Twenty-four cubic centimeters of a 2 per cent solution of Niagara sky blue injected while the plasma still contained large quantities of brilliant vital red (about 400 mg. dye per liter plasma). Samples of blood taken during the next 4 days were analyzed for their content of Niagara sky blue.

The results of all of these analyses are shown in Table 31.

Experiment 46. Dog 25–35, Male, Setter, 18 kg.

March 30, 1927. Twenty cubic centimeters of a 2 per cent solution of Niagara sky blue injected intravenously. During the next 4 days samples of plasma were collected for dye analysis.

May 4, 1927. Plasma found to be free of dye. Intravenous injection of 20 cc. of a 2 per cent solution of brilliant vital red together with 20. cc. of a 2 per cent solution of Niagara sky blue. Samples were taken for analysis at intervals during the next 4 days.

The results of the analyses are shown in Table 31.

Experiment 47. Dog 25–16. Airedale, 19 kg.

January 19, 1927. Intravenous injection of 20 cc. of a 2 per cent solution of Niagara sky blue. The concentration of dye in the plasma was determined at intervals during the next 4 days.

February 11, 1927. The plasma was free of dye. Intravenous injection consisting of 20 cc. of 2 per cent brilliant vital red along with 20 cc. of 2 per cent Niagara sky blue. Analyses of Niagara sky blue in the plasma at intervals during the next 4 days.

May 3 to 11th. Daily intravenous injections of 20 cc. of a 2 per cent solution of brilliant vital red.

TABLE 31

	Mg. Niagara sky blue per liter plasma after					
	5 min.	1 hour	6 hours	24 hours	48 hours	96 hours
Experiment 45						
Niagara sky blue alone	360	240	170	46	32	22
Niagara sky blue after chronic staining with brilliant vital red	310	210	130	43	30	16
Niagara sky blue in presence of brilliant vital red simultaneously injected	290	210	115	50	43	30
Experiment 46						
Niagara sky blue alone	340	190	81	36	17	10
Niagara sky blue in presence of brilliant vital red simultaneously injected	335	205	79	39	20	14
Experiment 47						
Niagara sky blue alone	330	195	90	44	18	12
Niagara sky blue after chronic staining with brilliant vital red	320	190	94	30	14	9
Niagara sky blue in presence of brilliant vital red simultaneously injected	300	192	99	45	23	15
Experiment 48						
Niagara sky blue alone	320	175	75	40	25	12
Niagara sky blue after chronic staining with brilliant vital red	275	180	91	59	23	10
Niagara sky blue in presence of brilliant vital red simultaneously injected	303	175	91	65	38	11

May 13. The dog is deeply stained and the plasma contains large amounts of the red dye (about 390 mg. dye per liter plasma). Twenty cubic centimeters of a 2 per cent solution of Niagara sky blue injected intravenously. Analyses were made at intervals during the next 4 days to determine the amount of the blue dye in the plasma.

The results of all the analyses are recorded in Table 31.

Experiment 48. Dog 24–74. Female, Shepherd, 28 kg.

January 20, 1927. Intravenous injection of 30 cc. of a 2 per cent solution of Niagara sky blue. The concentration of dye in the plasma was determined at intervals during the next 4 days.

February 15, 1927. The plasma was free of dye. Intravenous injection consisting of 30 cc. of a 2 per cent solution of brilliant vital red along with 30 cc.

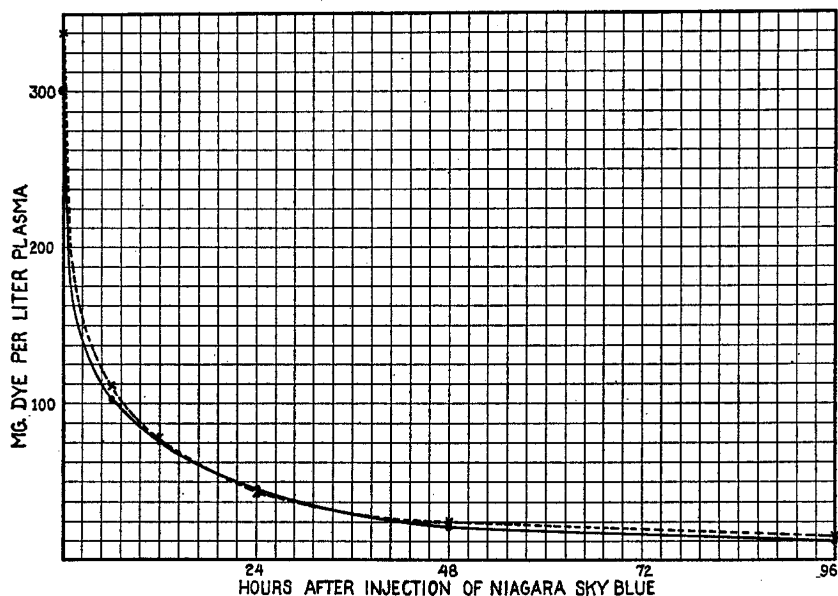


CHART 3A. Concentration of Niagara sky blue (average of 3 dogs). Interrupted line = unstained dogs. Continuous line = dogs previously stained with brilliant vital red.

of a 2 per cent solution of Niagara sky blue. Analyses of Niagara sky blue in the plasma at intervals during the next 4 days.

April 30 to May 8th inclusive, daily injections consisting of 30 cc. of 2 per cent solution of brilliant vital red.

May 10. The dog is deeply stained and the plasma contains large amounts of dye (about 320 mg. dye per liter plasma). Thirty cubic centimeters of a 2 per cent solution of Niagara sky blue injected intravenously. Analyses were made at intervals during the next 4 days to determine how rapidly the Niagara sky blue left the blood stream.

The results of all of the analyses are shown in Table 31.

Samples of centrifuged blood taken 5 minutes after an intravenous injection of brilliant vital red show plasma which is of a red color. If the dye present be measured colorimetrically it will be found that the amount present is directly proportional to the amount injected and inversely proportional to the total plasma volume of the animal. Similarly, an injection of Niagara sky blue imparts a bluish color to the plasma. Like brilliant vital red, this dye leaves the blood stream,

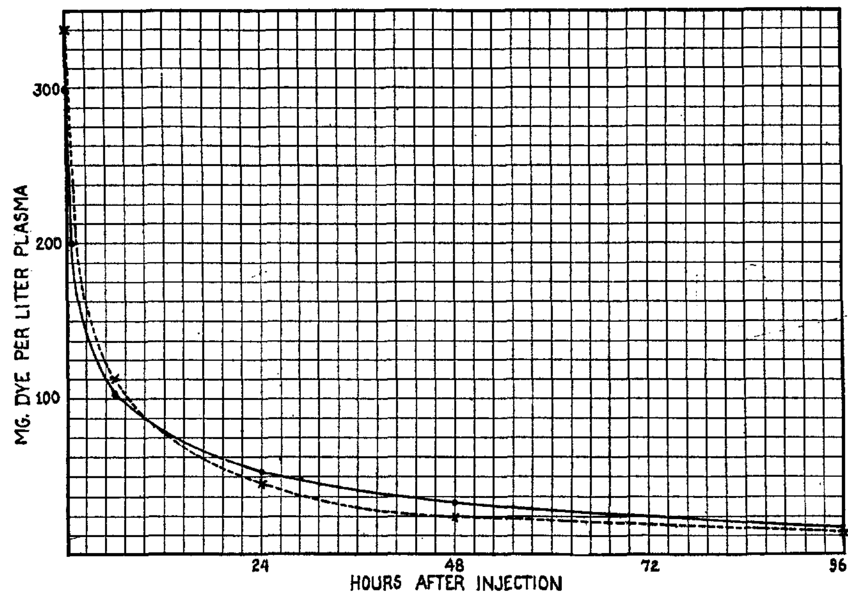


CHART 3B. Concentration of Niagara sky blue (average of 3 dogs). Interrupted line = Niagara sky blue injected alone. Continuous line = Niagara sky blue injected simultaneously with brilliant vital red.

and within 48 hours most of it is lodged in specialized phagocytic cells in various parts of the body. The rapidity with which each dye leaves the blood stream is somewhat different for each dye, and there are small individual differences in animals of the same species. With a given dye and under normal conditions the form of this curve is remarkably constant for a given animal.

We have given a series of four dogs an initial standardizing intravenous injection of Niagara sky blue. In each case the concentration

of dye in the plasma was measured five minutes later and again at intervals during the next 72 hours. A number of weeks were then allowed to elapse in order that the body might rid itself of the dye and be in a position to react normally once more. Each animal now received eight large daily injections of a two per cent solution of brilliant vital red. The tissues became progressively more red until staining was finally quite intense. To ascertain whether this red staining of the tissues reduces the ability of the latter to take up the blue dye from the plasma we then made an injection of the latter. We note from Chart 3A that, as in the original control experiment, the blue dye rises abruptly to a level of about 300 mg. per liter. From this point on until the end of the experiment 96 hours later the two curves are almost identical, showing quite unmistakably that these red-stained tissues will take up the blue dye just as effectively and as rapidly as do unstained tissues.

Some critic may wish to urge the point that some original phagocytic inhibition may have worn off before the test dose of blue dye was given. Indeed, it is held by many that dye-laden phagocytes may multiply by cell division or that new phagocytes may form from connective tissues with great rapidity and that these newly formed cells may take over the lost function of the stained cells, and thereby compensate for their deficiency. This point of view is thoroughly discussed by Aschoff (4). We must agree that some such increase in cells does occur when heavy injections are made repeatedly. We have also confirmed older observations that the individual phagocytes increase in size as they take up dye. But these data, presented in a paper to follow (2), include also some observations on double staining with these two dyes. It can be shown that when the two dyes are given in successive periods, the phagocytes contain granules of red and granules of blue dye, side by side. Very rarely indeed do we see cells which contain only one type of dye as we might expect if great numbers of new phagocytes were formed in the period of observation. The separate storage of materials in different cells reported by others need not signify that newly formed cells have been formed to take up the second substance; more likely is the view that the two substances used differed so widely that they simply found storage in cells which differed somewhat in type or in accessibility to the circulating blood.

We feel that purely morphological studies of these questions give only qualitative notions about the importance of cell multiplication in compensating for a hypothetical blockade. We are presenting certain physiological data which bear upon this point. The experiments were performed on the same dogs which we used in the experiments presented above. This second part of each experiment consisted in an effort to determine whether the tissues would take up Niagara sky blue at the normal rate if they were simultaneously busied with the phagocytosis of brilliant vital red. The animals were free of dye at the beginning of the experiment, but when the two dyes were mixed and injected we note that they gradually disappear from the plasma and become lodged largely within the tissues. It was thought that as the dyes accumulate together within the cells we might find a mutual antagonism—in other words, some evidence of failure of the cells to perform the double task of removing two dyes, each at its own normal speed. The fact that no such delay was observed is especially significant in these acute experiments. If phagocytic inhibition is to be observed at all we might expect surely to see it in these early stages of staining before cell multiplication could compensate for the filling of the cells with dye. If cell multiplication is responsible for the maintenance of phagocytic efficiency of the tissues we must be prepared to admit that the response is almost instantaneous—that there is little or no lag between incipient inhibition and compensatory multiplication and enlargement of cells.

In carrying out the experiments of which we have just spoken the two dyes were mixed in equal amounts and the mixture was injected into the blood stream. By means of the spectrophotometer we were able to measure the amount of each dye in the purple plasma which resulted. Samples of plasma were taken at intervals over a 72 hour period. From a technical point of view it is rather difficult to make precise measurements of brilliant vital red in this purple plasma, but the measurement of Niagara sky blue is much more easy and exact, for there is very little overlapping of the absorption bands at the red end of the spectrum where the blue dye must be measured. Furthermore the slight jaundice which sometimes develops does not interfere with the measurement of the blue dye as much as with the measurement of the red one. The concentration of the blue dye is shown in

Table 31 and graphically in Chart 3B. In this chart is also shown the normal control curve obtained some weeks previously by means of a simple injection of the blue dye alone. The two curves are almost identical throughout. The very slight retardation in elimination in the presence of the red dye is probably within the limits of experimental error. Like the experiments cited above, this experiment shows that a dye will pass from plasma into tissues at its own normal rate regardless of the presence of another dye. It is quite apparent that the tissues take up each dye in an independent manner.

DISCUSSION

We have already cited articles in which the reader will find discussions of the literature of "blockade" of the reticulo-endothelial system. It requires great generosity to admit much of the evidence which has been advanced in favor of the concept. We cannot but feel that much of the data is totally irrelevant. Changes in immune reactions, in fat metabolism, in pigment metabolism, and so forth, may well be associated with the injection of some foreign substance or with the use of some poison, and by some this is offered as evidence that the reticulo-endothelial system is blocked. Curiously enough this same evidence is offered by others to prove that the reticulo-endothelial system is concerned with immune reactions, fat or pigment metabolism. Obviously neither contention is built on firm foundation. Such rude procedures as these must cause great disturbances in the delicately regulated organism. Important changes in parenchymatous organs, changes in excretory activity of various organs are important considerations, and in our enthusiasm for the reticulo-endothelial system we must not neglect these other factors.

The rate at which dye leaves the blood stream depends upon excretion by liver and kidneys as well as upon the activity of the body phagocytes, nor, as we have shown, is it possible to disregard excretory activity by assuming it to be proportional to dosage. Such excretory activity must be measured carefully before we can assign to the phagocytes their proper importance in freeing the blood stream of dye, or of other materials. It is astonishing to see how generally this elementary precaution has been neglected. We have made some

progress in a study of the rate at which the liver excretes brilliant vital red, and certain of the data have been presented in the preceding paper. Unfortunately, Niagara sky blue is partially decolorized on reaching the bile, and for this reason it has not been possible to follow its elimination by the liver. The experiments presented above show that this dye leaves the blood stream at a normal rate, even though the body contains large amounts of red dye, and we have offered this as evidence that such red-stained tissues will take up the blue dye at a normal rate. Had the experiment turned out differently, and had we observed undue retention of the blue dye in the plasma, we would have been faced with the necessity of ascertaining whether defective phagocytosis or defective liver elimination was to blame, but since elimination is observed to be quite normal we do not need to postulate defective liver excretion. Indeed, if this could be shown to exist in this case, we would be forced to conclude that the red-stained tissues take up the blue dye *more* readily than normal, but there is no evidence to indicate that this is true, though this rather remote theoretical possibility must be kept in mind.

All things considered, we feel that brilliant vital red and Niagara sky blue are particularly well suited to studies on "blockade." They are not readily precipitated from solution by salts common in plasma or other body fluids and this is more than can be said of many substances which have been employed by others. The carbon of India ink is precipitated almost the moment it comes into contact with the plasma, and the rate at which these particles are filtered out of circulation gives us no clue about the activity of the phagocytic system. This same objection may be raised against the use of colloidal metals against the use of emulsions of fats and other substances.

The dyes we have used are free of these objections and we may confine our attention to storage by the tissues and to excretion from the body. These dyes are so much alike that they are taken up and stored within the same identical cells. With double staining experiments it is very rare to find cells which contain only one of the two dyes injected (2). It is quite otherwise when substances of widely different sort are used in combination. It is well known that the carbon particles of India ink do not pass readily through the capillary

walls. They soon become lodged within the phagocytes which are most directly related to the blood streams. We see them particularly in the Kupffer cells of the liver and in certain cells of the spleen. The enormous group of phagocytes in lymph nodes and in the connective tissues generally receive little or none of the carbon until a long period of time has elapsed. With such a substance there is no possibility of "blocking" more than a small part of the reticulo-endothelial system. One might well expect a normal response toward dyes and toward many other substances which readily reach cells which never come in contact with the ink. From such considerations we see the need of microscopic studies as controls in order that we may know at all times just which cells are concerned in any given process.

SUMMARY

When large amounts of brilliant vital red are injected into the blood stream of dogs, the dye is gradually removed from circulation, and most of it is deposited in numerous phagocytic cells which are scattered throughout various organs and tissues. The dye occurs largely in the form of tiny red granules crowded together in the cytoplasm of these cells.

If Niagara sky blue, a closely related dyestuff, is injected, it too is taken up and stored in these same cells. It is shown that the presence of red dye in the tissues does not inhibit the cells from taking up the blue one.

The normal ability of the phagocytes to take up Niagara sky blue is observed also when this dye is injected *simultaneously* with brilliant vital red. This normal response toward the blue dye is seen even though the phagocytes are busied at the same time in the process of engulfing and storing the red dye.

These experiments show that it is difficult if not impossible to "block" the cells with one dye so that their ability to take up another is even slightly impaired.

The two dyes employed in these studies are shown to be particularly suitable for experiments of the sort here reported.

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